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Joint effects of binary and multi-component, uniform and non-uniform mixtures assayed in our microbial toxicity studies were found to be simply additive, or essentially simply additive. These results are in agreement with the conclusions reported in the literature on fish toxicity studies. Using QSAR models to predict single chemical toxicity and assuming perfect simple additivity, concentrations of the components in mixtures that would cause 50% inhibition were predicted. These predicted conentrations agreed well with the measured values over nearly three orders of magnitude with r2=0.80 at p=0.0001 for 610 sets of data points from 40 different mixtures on two different microorganisms. The overall average factor of error of these predictions was 1.82. The results of this study provide an impetus to utilize the large number of single chemical QSAR models reported in the literature by other researchers in predicting joint effects in the aquatic toxicology and ecotoxicological fields.

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MICROBIAL TOXICITY OF MIXTURES OF ORGANIC CHEMICALS: MODELING AND VALIDATION FOR NON-UNIFORM MIXTURES

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1.0 INTRODUCTION

Toxicity of synthetic organic chemicals (SOCs) to microorganisms is an important consideration in assessing the chemicals' environmental impacts against their economic benefits. Microorganisms play important roles in several environmental processes, both natural and engineered. In natural systems, microorganisms drive the nutrient cycle and are also at the base of the food chain. In engineered systems, microbial processes offer cost-effective options to control and mitigate environmental pollution by SOCs. In addition, their response to SOCs may be extrapolated to higher life forms, whose testing is more expensive and elaborate (Cronin et al., 1991).

Traditionally, an assortment of microorganisms is utilized to biomineralize municipal and domestic wastewaters. These wastes are mainly composed of readily biodegradable organic chemicals; however, due to rapid industrialization, several SOCs at toxic levels have now been detected in municipal sewer lines resulting in plant upsets and discharge permit violations (Volskay and Grady, 1988). Since most municipal wastewater treatment plants were not originally designed to receive such SOCs, plant operators and regulatory agencies are concerned about the toxic effects of SOCs to microorganisms. Advance knowledge of toxicity of SOCs can benefit plant operators in: optimizing plant operation; setting pretreatment standards; establishing sewer discharge permits to safeguard the plant and to protect receiving water quality; and in waste load allocations.

Microbial processes have also emerged to be feasible technologies for treating industrial and hazardous wastes and for remediating groundwaters and soils contaminated with SOCs. While several SOCs have been demonstrated to be amenable to biological transformations into less harmful end products, their success is severely limited by the toxic and inhibitory threshold levels of the SOCs. Prior knowledge of toxicity of SOCs may be of benefit in these applications too.

Even though numerous toxicological studies on higher aquatic life forms have been reported in the literature, systematic toxicity assays of SOCs on environmentally relevant microorganisms utilized in waste mineralization have been undertaken only recently (Blum and Speece, 1990; Tang et. al., 1992; Nirmalakhandan et al., 1993; and Sun et al., 1994). Apart from the numerical toxicity values, these data sets also encode valuable toxicological information: interspecies correlations; organism sensitivity; chemical structure-toxicity and chemical property-toxicity relationships etc. Since the resources available for comprehensive and exhaustive toxicity testing of all the SOCs in current use are severely limited, it is prudent to maximize the knowledge that can be gleaned from available data upon which considerable resources have been expended.

1.1 Structure/Property Activity Relationships

Quantitative structure-activity relationship (SAR) and property-activity relationship (PAR) techniques have emerged as rational tools to explore available test data for extracting the maximum possible information from them and for developing predictive models. The premise of these

techniques is that properties and activities of organic molecules are well correlated to their molecular structures and basic physical/chemical properties. Using these techniques, it is now possible to derive models from available test results that could predict reliable data for related, untested chemicals. While the state-of-the-art SAR and PAR techniques cannot yet completely replace experimental testing, they are being extensively used to complement test data in: designing new chemicals with desired characteristics (Cramer, 1980); environmental assessments (Borman, 1990); screening and grouping similar chemicals for collective evaluation (Clements et al., 1993); prioritizing testing (Zeeman et al., 1993); in classifying mechanisms and modes of toxic action (Bradbury, 1994); and, in predicting joint toxicity in mixtures of chemicals (Nirmalakhandan et al., 1993; Hall et al., 1996; Xu and Nirmalakhandan, 1997).

Such models can conserve the limited resources available for toxicity testing in a timely manner and guide the regulatory risk assessment process in a rational and scientific manner. Recognizing these advantages, the US Environmental Protection Agency (EPA), for example, has adapted over 50 PAR models for use on a daily basis for estimating toxicity of untested SOCs to several different fish species (EPA-560/6-88-001). In addition, EPA uses SAR methodology to assess the environmental fate, hazards, and risks of proposed new industrial chemicals prior to their commercial manufacture (Zeeman et al., 1993). The OECD and the German Federal Environmental Agency also use SAR in similar regulatory actions (Fiedler et al., 1990).

In this research, it is hypothesized that SAR techniques could be applied to predict joint toxic effects of mixtures of SOCs on microorganisms. Even though the toxic effects of multiple chemicals acting jointly on aquatic life forms have been the subject of considerable research in the past three decades, such effects on microorganisms have not been studied until now.

1.2 Joint Effects in Mixtures of Chemicals

The study of joint effects originated with the analysis of the effect of two chemicals in binary mixtures. Plackett and Hewlett (1967) identified four types of joint effects: similar vs. dissimilar depending on whether the sites of action and the modes of primary action of the two chemicals are the same or different; and interactive vs. non-interactive, depending on whether one chemical does or does not influence the biological action of the other. If the response of the organism is produced by a combination of the two chemicals, then they are said to exert joint action. This joint action can be further classified into simply additive, more than additive, and less than additive. When this scheme is applied to multi-component mixtures, the analysis becomes complex, because the joint actions of different pairs may fall into different types of joint action.

In quantifying the effects of components in mixtures, the concept of toxic unit is often used. It is defined as:

$$TU_i = \frac{z_i}{Z_i} \qquad (1)$$

where, z_i is the concentration of a component i in a mixture that causes a certain response, and Z_i is its concentration causing the same response when acting singly. In microbial toxicity, this response could be 50% inhibition of respiration rate. If the TUs of all the components in a mixture are equal, then the mixture is referred to as an equitoxic or a uniform mixture.

Using the TU concept, ecotoxicologists have proposed alternate schemes to characterize the degree of joint action of multiple chemicals acting together. In one scheme, the sum of the TUs of the components, M (i.e. $M = \sum TU_i$), is used as an index to categorize the type of joint action: if $M = 1$, the components are simply additive (also referred to as concentration addition); if $M < 1$, more than additive; and, if $M > 1$, less than additive. Hermens et al (1985) evaluated literature toxicity data on fish and found average $M = \sum TU_i = 0.9$ in mixtures of 50 non-reactive chemicals; and average $M = \sum TU_i = 1.1$ in 17-component mixtures. They concluded that the chemicals acted together by simple addition since M values were "very close to 1".

In another scheme, proposed by Marking (1977), an additive index, AI, is used as the index where,

$$AI = \frac{1}{M} - 1 \quad \text{if } M \leq 1$$
$$AI = 1 - M \quad \text{if } M > 1 \qquad (2)$$

According to this scheme, when $AI = 0$, components are simply additive; if $AI > 0$, then more than additive; and, if $AI < 0$, less than additive. Lewis and Perry (1981) applied this scheme to analyze joint effects of equitoxic mixtures of three chemicals on bluegills and found AI values ranging from 0.30 to -1.23. Even though several AI values in their studies deviated significantly from 0, they concluded that the chemicals acted by simple addition, based on the average AI of 0.05.

Another scheme proposed by Konemann (1981) uses a mixture toxicity index, MTI, defined as:

$$MTI = 1 - \frac{\log M}{\log M_o} \qquad (3)$$

where $M_o = M \div$ the largest TU_i in the mixture. In this scheme, MTI = 1 implies simply additive; MTI = 0 independent action; MTI < 0 antagonism; MTI > 1 the supra-addition; and 1> MTI >0, partial addition. Broderius and Kahl (1985) used this scheme to analyze joint effects of several equitoxic 7-, 14-, and 21-component mixtures, and concluded simple additivity with MTI values ranging from 0.93 to 1.06. Hermens et al (1984) evaluated joint effects of 14 miscellaneous chemicals to *Daphnia magna* and concluded simple addition, with an average MTI of 0.95.

Christensen and Chen (1989) have proposed an index defined as the similarity parameter, λ, for use in mixture toxicity analysis. According to this formulation, for an n-component mixture,

$$\sum_{1}^{n} (TU_i)^{\frac{1}{\lambda}} = 1 \qquad (4)$$

For noninteractive toxicity, λ is restricted to lie between 0 and 1, and when $\lambda = 1$, the joint action is identified as simply additive. When $\lambda > 1$, interactive and more than additive is implied. Christensen and Chen (1989) used binary toxicity data from the literature and showed that, for chemicals acting by similar mechanisms, λ ranged from 0.87 to 1.23, and concluded simple additivity.

1.3 Joint Effects by Simple Addition

It is of interest to note the consensus among aquatic toxicologists that most organic chemicals act jointly by simple addition. Hermens and coworkers (1984, 1985) found toxicities of 3 mixtures of anilines (n = 6, 11, and 17) to guppies to be simply additive with MTI values of 0.95, 0.96, and 0.97. In another study of mixtures of 14 miscellaneous chemicals to Daphnia magna, Hermens et al. (1984) reported that the joint effects were simply additive with MTI of 0.95. They also tested mixtures containing from 5 up to 50 chemicals on Daphnia magna, and again found the simply additive model valid with MTI values ranging from 0.7 to 1.05. Broderius and Kahl (1985) evaluated the joint effects on fathead minnow, and reported that acute toxicity of binary and multiple-component mixtures containing up to 21 constituents to be simply additive, with M ranging from 0.87 to 1.23; AI from -0.233 to 0.149; and MTI from 0.932 to 1.200. Wolf et al. (1988) reported similar findings about the simple addition model when tested on the joint effects of wide range of chemicals on Daphnia magna. For mixtures consisting of up to 25 chemicals, their M values ranged from 1.04 to 1.20; and MTI from 0.921 to 0.988. Broderius et al (1995) evaluated toxicity to fathead minnow of binary mixtures of 46 industrial chemicals and concluded that the chemicals acted primarily by simple addition and interactive toxicity was not common in their experiments.

If the joint effects of a set of chemicals in a mixture can be accepted to be simply additive, then their concentrations in any mixture that would result in a certain response can be readily estimated from their respective individual concentrations causing the same response, when acting singly. The practical utility of this deduction can be further enhanced by incorporating Quantitative Structure Activity Relationship (QSAR) models to estimate the individual IC50 values directly from the molecular structures of the components. This provides a strong impetus to develop QSAR models for single chemical toxicity and to validate their usage in predicting joint effects in mixtures of chemicals.

2.0 OBJECTIVES OF THIS PROJECT

During the first phase of this project, it was documented that QSAR techniques could be applied successfully to predict joint toxic effects of uniform mixtures. In this second phase, additional chemicals are assayed to demonstrate the applicability of the proposed approach to "new" chemicals that were not included in the QSAR model development. In addition, the approach is now demonstrated on non-uniform mixtures as well. The following are the specific objectives of the second phase:

1. Development of tools to analyze uniform and non-uniform mixture results
2. Development and validation of an approach to predict joint effects in non-uniform mixtures
3. Compare the predictive ability of the proposed approach with that of other models reported in the literature.

Towards meeting the above objectives, the following modeling and experimental tasks are undertaken in this second phase:

2.1 Modeling tasks:

1. Development of a statistically valid testing procedure to confirm simple addition in uniform and non-uniform mixtures
2. Development of a QSAR-based modeling procedure to predict concentrations of chemicals in uniform and non-uniform mixtures that would jointly cause 50% inhibition of respiration.
3. Compare the modeling approach developed in this research against other SAR/PAR approaches reported in the literature for single chemical microbial toxicity.

2.2 Experimental tasks:

1. Determination of single chemical IC50 values and their reproducibility with four different runs for each experiment for a new set of test chemicals for two microbial cultures: Polytox and activated sludge
2. Determination of IC50 values for six 6-component and eight 8-component non-uniform mixtures of the new set of test chemicals for two microbial cultures: Polytox and activated sludge.

Details of the above tasks are presented in the next section.

3.0 MODELING TASKS

3.1 Testing For Simple Addition

When one wishes to use the above schemes to determine whether a specified set of chemicals would act together by simple addition or not, statistically valid "acceptance limits" have to be assigned to the indexes M, AI, MTI, or λ. These limits should account for the variances due to experimental errors and the reproducibilities associated with the z_i and Z_i values. This would enable end users to analyze and estimate multi-component mixture toxicity with a known degree of reliability. Marking (1977) has proposed a simple method to assign a range to the additivity index. According to this method, the 95% confidence intervals for the IC50 values are substituted in the formulas for determining AI. The lower and upper limits of IC50 values are used to get a range and if that range included zero, additive toxicity is assumed to be valid. In this paper, two approaches are proposed to assign acceptable ranges based on TU and λ to conclude simple additivity in non-uniform mixtures.

3.1.1 Range based on TU

In a mixture with n components, an acceptance range for TU of the n^{th} chemical, TU_n, that would induce 50% inhibition in the presence of known TUs of the other (n-1) chemicals has to be established. This may be obtained from the regression of % inhibition vs. TU_n data, by assigning a confidence interval to the inverse predicted TU_n corresponding to 50% inhibition. This interval is obtained from Bethea et al., (1985):

$$\hat{x} \pm \varepsilon = \bar{x} + \frac{\hat{\beta}_1 (y_0 - \bar{y})}{\phi} \pm \frac{t\hat{\sigma}}{\phi} \left[c(y_0 - \bar{y})^2 + \phi \frac{n+1}{n} \right]^{\frac{1}{2}} \qquad (5)$$

where,

$$c = \frac{1}{\sum_1^n (x_i - \bar{x})^2}$$

$$\phi = \hat{\beta}_1^{\,2} - c t^2 \hat{\sigma}^2$$

and, $t = t_{n-2, \frac{\alpha}{2}}$

The expected TU_n for simple additivity can be found from

$$TU_n = \sum_1^n TU_i - \sum_1^{n-1} TU_i = 1 - \sum_1^{n-1} TU_i \qquad (6)$$

If this expected TU_n falls within the range calculated from eq (5), then the components in that mixture could be considered simply additive at the selected confidence level, α.

3.1.2 Range based on λ

The range for λ may be determined from the variance in the TUi (= zi/Zi). The variance in TUi is caused by the variances in zi and Zi which can be estimated from

$$\text{Var TU}_i = \text{Var}\left(\frac{z_i}{Z_i}\right) = \left(\frac{\mu_{z_i}}{\mu_{Z_i}}\right)^2 \left[\frac{\text{Var } z_i}{\mu^2_{z_i}} + \frac{\text{Var } Z_i}{\mu^2_{Z_i}}\right] \qquad (7)$$

where, μ_{zi} is the mean of the dosage of any chemical added to the reactor and μ_{Zi} is the mean of the IC50 of that chemical; Var z_i is the variance in the concentration of the dosage of the chemical; and, Var Z_i is the variance in the IC50 value of that chemical. Var z_i depends on the error in the volumes of the chemical dosages. Var Z_i depends on the chemical being tested and the reproducibility in the procedure used for the IC50 measurements. Based on syringe manufacturers' data, a 0.5% error in the syringe volumes is typical; thus the average Var z_i can be estimated to be around 0.5 mg/L. For most chemicals this can be considered negligible compared to the Var Z_i. For example, based on reproducibility tests performed by us on 4 chemicals a standard deviation of 16.4 mg/L was found to be typical (Nirmalakhandan, et al., 1994). Thus eq (7) can be reduced to the following form:

$$\text{Var TU}_i = \text{Var}\left(\frac{z_i}{Z_i}\right) = \frac{\mu^2_{z_i}}{\mu^4_{Z_i}} \text{Var } Z_i \qquad (8)$$

The square root of the above Var TU_i gives the standard deviation of TU_i, which when multiplied by 2 can yield a 95% confidence interval for TU_i. These upper and lower values for TU_i can then be substituted in eq 4 and solved by trial and error to yield an acceptance range for λ. If this range included 1, then the components in the mixture could be considered simply additive at the 95% confidence level.

3.2 Prediction of Concentrations in Mixtures Causing 50% Inhibition

The method being proposed is based on the following two premises:

- that simple addition is an adequate mechanism by which joint effects could be quantified;
- that SAR models can adequately predict single chemical toxicity.

With these two premises, the concentrations of components in a mixture that would jointly cause the desired end point can be predicted from their toxic units to uniform and nonuniform mixtures as follows:

3.2.1 Application to uniform mixtures:

In a uniform mixture of n chemicals, toxic unit of each component will be the same. In such cases, assuming simple addition, 50% inhibition would occur when

$$\sum_{i=1}^{n} TU_i = n * TU_i = 1 \qquad (9)$$

giving

$$TU_i = \frac{1}{n} \qquad (10)$$

Therefore, the concentration, C_i, of any component, i, in the mixture that would contribute to 50% inhibition can be found based on its single chemical IC_{50} value from:

$$C_i = \frac{IC_{50,i}}{n} \qquad (11)$$

The $IC_{50,i}$ values in turn may be predicted directly from the molecular structures of the components using QSAR models, such as eq 5.

3.2.2 Application to non-uniform mixtures:

In the case of non-uniform mixtures, if the toxic units or proportions of (n-1) components are known, the concentration of the n^{th} chemical (C_n) that would induce the end point along with the other (n-1) chemicals can be predicted from:

$$C_n = IC_{50,n} * \left[1 - \sum_{i=1}^{n-1} TU_i \right] \qquad (12)$$

Again, $IC_{50,n}$ values may be predicted directly from the molecular structure of the n^{th} component using SAR models.

4.0 EXPERIMENTAL TASKS

In this research, toxicity was quantified by IC50, the dose of the test chemical that inhibited microbial respiration by 50% compared to an undosed control. A total of 16 chemicals were chosen to form the testing set so as to contain a mix of structural and elemental features that were represented only individually in the original training sets used in developing the SAR/PAR models. IC50 values for this testing set of 16 chemicals on Polytox and activated sludge were determined in this study using a computer-interfaced, 12-reactor, Comput-OX Respirometer (N-CON Systems, Crawford, GA). Details of the experimental system and the test procedures for determining IC50 values for single chemicals and uniform mixtures have been presented in detail in the previous report and in several of our publications (Nirmalakhandan et al., 1993; Hall et al., 1996).

4.1 Determination of IC50 in non-uniform mixtures

In one series of non-uniform mixture tests, six organic chemicals were mixed in different Toxic Units to form six different non-uniform mixtures (n = 6). In another series, eight organic chemicals were mixed in different Toxic Units to form eight different non-uniform mixtures (n = 8). The composition and the TUs of the components of the different mixtures are listed in Table II. Toxicity assays were conducted using a 12-reactor computer interfaced Comput-Ox Respirometer Two reactors were used as controls and the remaining reactors were dosed with the mixtures. For each test mixture of n chemicals, the TU of (n-1) chemicals were kept constant; the n^{th} chemical was added at various TU_n of 0.1, 0.2, 0.3. 0.4 and 0.5 to the mixture. Oxygen uptake rates of the reactors dosed with the different mixtures were compared against those of the control reactors to determine the % inhibition. Then, from plots of % inhibition vs. TU_n, the $TU_{n,50\%}$ corresponding to 50% inhibition was obtained. Each mixture was tested three times to obtain triplicate $TU_{n,50\%}$ values, which were then used to determine the average $\sum TU_i$ for that mixture.

5.0 RESULTS

5.1 Single chemical QSAR models

The following single chemical QSAR models for different chemical types and test cultures derived during the first phase of the project (Sun, 1993; Hall et al., 1996) are validated in this study. The chemicals used in the first phase in developing these models are listed in Table I followed by the 16 chemicals assayed in this second phase for validation purposes.

QSAR models for Polytox cultures:

Alcohols, ketones and esters:

$$\log IC_{50}\ [mM/L] = 3.69 - 0.90\ {}^{1}\chi^{v} \quad (13)$$
$$n = 14;\ r^2 = 0.910;\ \text{RMS residual} = 0.25;\ p = 0.0001$$

Alkanes:

$$\log IC_{50}\ [mM/L] = 1.85 - 0.76\ {}^{1}\chi^{v} \quad (14)$$
$$n = 11;\ r^2 = 0.924;\ \text{RMS residual} = 0.13;\ p = 0.0001$$

Amines and acids:

$$\log IC_{50}\ [mM/L] = 1.18 - 0.51\ {}^{1}\chi^{v} \quad (15)$$
$$n = 11;\ r^2 = 0.902;\ \text{RMS residual} = 0.123;\ p = 0.0001$$

Aromatics:

$$\log IC_{50}\ [mM/L] = 3.25 - 1.13\ {}^{1}\chi^{v} \quad (16)$$
$$n = 11;\ r^2 = 0.788;\ \text{RMS residual} = 0.278;\ p = 0.0003$$

Halogenated aliphatics:

$$\log IC_{50}\ [mM/L] = 2.67 - 0.44\ {}^{0}\chi^{v} \quad (17)$$
$$n = 12;\ r^2 = 0.885;\ \text{RMS residual} = 0.143;\ p = 0.0001$$

QSAR models for Activated sludge cultures:

Alcohols, ketones and esters:

$$\log IC_{50}\ [mM/L] = 3.43 - 0.75\ {}^{1}\chi^{v} \quad (18)$$
$$n = 14;\ r^2 = 0.832;\ \text{RMS residual} = 0.30;\ p < 0.0001$$

Alkanes:

$$\log IC_{50}\ [mM/L] = 2.02 - 0.63\ {}^{1}\chi^{v} \quad (19)$$
$$n = 11;\ r^2 = 0.750;\ \text{RMS residual} = 0.23;\ p = 0.0006$$

Amines and acids:

$$\log IC_{50}\ [mM/L] = 0.89 - 0.29\ {}^{1}\chi^{v} \quad (20)$$
$$n = 11;\ r^2 = 0.629;\ \text{RMS residual} = 0.19;\ p = 0.0036$$

Aromatics:

$$\log IC_{50}\ [mM/L] = 3.54 - 1.28\ {}^{1}\chi^{v} \quad (21)$$
$$n = 12;\ r^2 = 0.660;\ \text{RMS residual} = 0.39;\ p = 0.0013$$

Halogenated aliphatics:

$$\log IC_{50}\ [mM/L] = 2.92 - 0.48\ {}^{0}\chi^{v} \quad (22)$$
$$n = 14;\ r^2 = 0.785;\ \text{RMS residual} = 0.22;\ p < 0.0001$$

TABLE I. Chemicals Assayed, Connectivity Indices, and Measured IC50 Values

ID #	Chemical name	Chemical family*	MCI**		Exp. IC50, [mg/L]	
			$^0\chi v$	$^1\chi v$	Polytox	A/S
1	Benzene	ARO	3.46	2.00	685	993
2	Toluene	ARO	4.38	2.41	207	292
3	Xylene	ARO	5.30	2.82	140	166
4	Ethylbenzene	ARO	5.09	2.97	220	222
5	Chlorobezene	ARO	4.52	2.47	350	155
6	1,2-Dichlorobezene	ARO	5.57	2.96	135	49
7	1,3-Dichlorobezene	ARO	5.57	2.95	40	63
8	1,4-Dichlorobezene	ARO	5.57	2.95	6	14
9	1,2,4-Trichlorobezene	ARO	6.63	3.43	23	35
10	2,4-Dimethyl phenol	ARO	5.67	2.96	240	224
11	Methylene chloride	HAL	2.97	1.60	1,750	1,994
12	Dibromomethane	HAL	4.63	2.77	1,110	1,572
13	Carbon tetrachloride	HAL	5.03	2.26	325	432
14	1,2-Dichloroethane	HAL	3.68	2.10	685	1,385
15	1,1,1-Trichloroethane	HAL	4.90	2.20	415	659
16	1,1,2,2-Tetrachloroethane	HAL	5.68	2.29	180	197
17	1,2-Dichloropropane	HAL	4.55	2.44	500	861
18	Bromochloromethane	HAL	3.80	2.19	1,800	2,672
19	Bromodichloromethane	HAL	4.80	2.44	90	249
20	Chlorodibromomethane	HAL	5.64	2.92	425	206
21	Ethylene dibromide	HAL	5.13	2.31	520	1,271
22	1,2-Dichloroethylene	HAL	3.42	1.64	350	1,249
23	Trichloroethylene	HAL	4.47	2.07	500	770
24	Tetrachloroethylene	HAL	5.53	2.51	175	299
25	Cyclohexane	ALK	4.24	3.00	74	133
26	Pentane	ALK	4.12	2.41	70	150
27	Hexane	ALK	4.82	2.91	38	47
28	Heptane	ALK	5.53	3.41	18	58
29	Octane	ALK	6.24	3.91	8	60
30	Bis(2-chloroethyl) ether	AKE	5.50	3.18	1,600	28,541
31	Ethanol	AKE	2.15	1.02	40,000	28,541
32	Propanol	AKE	2.86	1.52	7,200	10,875
33	Pentanol	AKE	4.27	2.52	2,325	3,528

Note: * ARO - Aromatics; HAL - Halogenated aliphatics; ALK - Alkanes; AKE - Alcohols, ketones and esters. ** MCI - Molecular Connectivity Index

TABLE I (Contd.)

ID #	Chemical name	Chemical family*	MCI**		Exp. IC50, [mg/L]	
			$0\chi v$	$1\chi v$	Polytox	A/S
34	Octanol	AKE	6.39	4.02	126	194
35	n-Butyl acetate	AKE	5.43	2.90	3,750	1,649
36	Isobutyl acetate	AKE	5.60	2.75	1,600	2,156
37	n-Amyl acetate	AKE	6.14	3.40	440	1,031
38	Ethyl acetate	AKE	4.02	1.90	5,400	5,427
39	Acetone	AKE	2.90	1.20	48,000	48,619
40	Methyl ethyl ketone	AKE	3.82	1.99	1,900	1,873
41	Methyl isobutyl ketone	AKE	5.19	2.62	2,600	2,811
42	Methyl n-propyl ketone	AKE	4.32	2.26	4,500	4,267
43	Cyclohexanone	AKE	4.44	2.41	3,750	5,452
44	n-Butyl amine	AMI	3.69	2.11	90	111
45	y-Butyl amine	AMI	4.07	1.78	85	90
46	Diethylamine	AMI	3.91	2.12	104	100
47	Acetic acid	AMI	2.35	0.93	287	299
48	Cyclohexylamine	AMI	4.69	2.64	60	103
49	Ethanolamine	AMI	2.43	1.22	160	115
50	Trithanolamine	AMI	6.03	3.39	900	741
51	2,2,2-Trichloroethanol	AKE	5.05	2.37	2,813	2,685
52	2,2-Dichloroethanol	AKE	3.99	2.03	8,047	8,836
53	1,2-Dichloro 2-methyl propane	HAL	5.47	2.72	744	635
54	1,2,3-Trichloropropane	HAL	5.39	3.07	534	564
55	Cyclopentane	ALK	3.54	2.50	129	164
56	1,1,2-Trichloroethane	HAL	4.68	2.51	726	1,023
57	1,3-Dichloropropene	HAL	4.12	2.19	274	369
58	m-Cresol	ARO	4.75	2.54	643	580
59	p-Cresol	ARO	4.75	2.54	833	522
60	2-Nitrophenol	ARO	4.09	2.17	993	318
61	4-Nitrophenol	ARO	4.09	2.25	431	125
62	2,4-Dinitrophenol	ARO	4.50	2.37	523	169
63	2,4-Dichlorophenol	ARO	5.94	3.09	166	73
64	2,3,4-Trichlorophenol	ARO	7.00	3.58	58	32
65	2,3,5-Trichlorophenol	ARO	7.00	3.57	66	32
66	2,4-Dinitrotoluene	ARO	5.05	2.65	530	199

Note: * ARO - Aromatics; HAL - Halogenated aliphatics; ALK - Alkanes; AKE - Alcohols, ketones and esters. ** MCI - Molecular Connectivity Index

5.1.1 Statistical Validity of the Models

In linear regression studies, it is preferred to have 10 to 20 cases per variable. Since the number of chemicals in three of the five subgroups in this study are near the lower limit, additional statistical criteria were examined to verify the validity of the above models. First, the significance of each of the models was evaluated based on "p" values. In toxicity studies, $p \leq 0.05$ is often considered "borderline statistically significant", $p < 0.01$ as "significant", and $p < 0.005$ as "highly significant". According to this guideline, all the above models could be considered highly significant. That is, all these models can be expected to predict toxicity of chemicals of similar structure better than that would be expected by pure chance alone.

Since a basic assumption in linear regression procedure is that the error terms in the resulting model are independent, the residuals were examined next. The residuals for all the chemicals were found to be randomly distributed when plotted against the fitted values and the fitted error is within a factor of two for 83% of the chemicals used in the model development. Standard normal probability plot of the residuals closely followed a linear pattern confirming normal distribution and the absence of any outliers. Serial autocorrelation within residuals close to 1 or -1 implying dependent cases is a common violation of the assumptions in linear regression. The serial autocorrelation in the above models ranged from -0.392 to 0.403, indicating minimal impact. This is further confirmed by the Durbin-Watson "d" values for the above models which ranged from 1.182 to 1.693. These d values are above the lower limit of ~1.0 for single variable models with 10 cases (Durbin and Watson, 1951). Thus, the above models can be considered robust and the utility of these models in predicting IC_{50} data may be of value.

5.2 Mixture Toxicity Results

In this report, single chemical, binary mixture, and multi-component uniform and nonuniform mixture toxicity data presented in our earlier works (Nirmalakhandan et al, 1994; Sun et al, 1994; Hall et al, 1995; Prakash et al, 1996; and Peace et al, 1997) are consolidated to illustrate the utility of the QSAR approach in estimating joint effects in mixtures. Additional new data on mixtures of chemicals not included in the QSAR model development are also used to validate the predictive ability of the proposed approach in nonuniform mixtures. A total of 66 organic chemicals (Table I) were tested in various binary and multiple combinations and in uniform and non-uniform proportions.

5.2.1 Simple additivity in microbial toxicity

Table II summarizes all the different types of mixtures assayed, their respective constituents, and the test cultures. Results of these tests were first analyzed for simple additivity. Table III summarizes the 95% confidence intervals for ΣTU, AI, MTI, and λ values for the different types of mixtures assayed. Since the 95% confidence interval for all the mixtures except the 10-component mixtures on A/S include the expected value of $\lambda = 1.0$, it is concluded that the tested chemicals all act by simple addition.

TABLE II. Types of Mixtures Assayed and their Constituents

Mixture ID #	Ref.	Mixture type	Components*	Cultures
6M1 to 6M6	a	6-chemical, non-uniform	51,52,53,54,55,56	Polytox
8M1 to 8M8	a	8-chemical, non-uniform	51,52,53,54,55,56,57,58	Polytox
6M1 to 6M10	a	6-chemical, non-uniform	51,52,53,54,55,56	A/S
8M1 to 8M8	a	8-chemical, non-uniform	51,52,53,54,55,56,57,58	A/S
8M1	b	8-chemical, non-uniform	12,30,32,33,35,36,40,41	Polytox
8M2	b	8-chemical, non-uniform	1,2,12,18,35,36,40,41	Polytox
8M3	b	8-chemical, non-uniform	22,23,32,33,35,36,40,41	Polytox
8M4	b	8-chemical, non-uniform	4,12,17,18,32,33,34,36	Polytox
8M5	b	8-chemical, non-uniform	4,10,18,22,23,32,33,36	Polytox
8M6	b	8-chemical, non-uniform	2,4,5,10,15,21,35,40	Polytox
8M1	c	8-chemical, uniform	12,30,32,33,35,36,40,41	A/S
8M2	c	8-chemical, uniform	1,2,12,18,35,36,40,41	A/S
8M3	c	8-chemical, uniform	22,23,32,33,35,36,40,41	A/S
8M4	c	8-chemical, uniform	4,12,17,18,32,33,34,36	A/S
8M5	c	8-chemical, uniform	4,10,18,22,23,32,33,36	A/S
8M6	c	8-chemical, uniform	2,4,5,10,15,21,35,40,	A/S
10M1	c	10-chemical, uniform	1,2,4,5,10,12,18,32,33,36	A/S
10M2	c	10-chemical, uniform	4,5,10,12,18,22,23,32,33,36	A/S
10M3	c	10-chemical, uniform	4,5,10,17,32,33,35,36,40,41	A/S
10M4	c	10-chemical, uniform	2,4,5,10,32,33,35,36,40,41	A/S
10M5	c	10-chemical, uniform	17,31,32,33,34,35,36,40,41,43	A/S
10M6	c	10-chemical, uniform	1,2,12,18,31,35,36,40,41,43	A/S
10M7	c	10-chemical, uniform	12,18,22,23,31,32,33,40,41,43	A/S
10M8	c	10-chemical, uniform	4,5,17,32,34,35,36,40,41,43	A/S
10M9	c	10-chemical, uniform	1,2,4,5,17,18,35,36,40,41	A/S
10M10	c	10-chemical, uniform	4,5,12,17,18,22,23,40,41,43	A/S

Note: a: This Study; b: Peace, 1995; c: Hall, 1995.

* Refer to ID# in Table I.

TABLE II (Contd.)

Mixture ID#	Ref.	Mixture type	Components*	Cultures
8M1	d	8-chemical, uniform	12,30,32,33,35,36,40,41	Polytox
8M2	d	8-chemical, uniform	1,2,12,18,35,36,40,41	Polytox
8M3	d	8-chemical, uniform	22,23,32,33,35,36,40,41	Polytox
8M4	d	8-chemical, uniform	4,12,17,18,32,33,34,36	Polytox
8M5	d	8-chemical, uniform	4,10,18,22,23,32,33,36	Polytox
8M6	d	8-chemical, uniform	2,4,5,10,15,21,35,40	Polytox
10M1	d	10-chemical, uniform	1,2,4,5,10,12,18,32,33,36	Polytox
10M2	d	10-chemical, uniform	4,5,10,12,18,22,23,32,33,36	Polytox
10M3	d	10-chemical, uniform	4,5,10,17,32,33,35,36,40,41	Polytox
10M4	d	10-chemical, uniform	2,4,5,10,32,33,35,36,40,41	Polytox
10M5	d	10-chemical, uniform	17,31,32,33,34,35,36,40,41,43	Polytox
10M6	d	10-chemical, uniform	1,2,12,18,31,35,36,40,41,43	Polytox
10M7	d	10-chemical, uniform	12,18,22,23,31,32,33,40,41,43	Polytox
10M8	d	10-chemical, uniform	4,5,17,23,34,35,36,40,41,43	Polytox
10M9	d	10-chemical, uniform	1,2,4,5,17,18,35,36,40,41	Polytox
10M10	d	10-chemical, uniform	4,5,12,17,18,22,23,40,41,43	Polytox
B1	e	Binary, non-uniform	5,34	Polytox
B2	e	Binary, non-uniform	34,37	Polytox
B3	e	Binary, non-uniform	10,34	Polytox
B4	e	Binary, non-uniform	16,34	Polytox
B5	e	Binary, non-uniform	34,44	Polytox
B6	e	Binary, non-uniform	34,49	Polytox
B7	e	Binary, non-uniform	20,34	Polytox
B8	e	Binary, non-uniform	24,34	Polytox
B9	e	Binary, non-uniform	34,43	Polytox

Note: d: Mohsin, 1993; e: Prakash, 1994

* Refer to ID# in Table I.

Table III: Analysis of joint effects in all the mixtures tested

Mixture Type	Test Cultures	Ref.*	Toxicity Indexes used in analysis			
			ΣTU_i	AI	MTI	λ
Non-uniform:						
6-chemical, non-uniform	Polytox	a	0.98 - 1.20	–0.16 - –0.04	0.87 - 0.96	0.646 - 1.246
8-chemical, non-uniform	Polytox	a	0.91 - 1.18	–0.14 - 0.10	0.88 - 1.07	0.768 - 1.168
6-chemical, non-uniform	A/S	a	0.93 - 1.11	–0.06 - 0.05	0.96 - 1.04	0.904 - 1.094
8-chemical, non-uniform	A/S	a	0.94 - 1.13	–0.12 - 0.04	0.91 - 1.02	0.859 - 1.121
8-chemical, non-uniform	Polytox	b	1.02 - 1.27	–0.27 - –0.02	1.02 - 1.25	0.835 - 1.053
Uniform:						
8-chemical, uniform	A/S	c	1.10 - 1.55	–0.55 - –0.10	0.79 - 0.95	0.774 - 1.014
10-chemical, uniform	A/S	c	1.50 - 1.91	–0.91 - –0.31	0.72 - 0.88	0.665 - 0.902
8-chemical, uniform	Polytox	d	0.92 - 1.21	–0.08 - 0.21	0.92 - 1.04	0.889 - 1.079
10-chemical, uniform	Polytox	d	0.90 - 1.31	–0.1 - 0.31	0.94 - 1.05	0.868 - 1.092
Binary:						
Binary, non-uniform	Polytox	e	0.69 - 1.51	–0.45 - 0.45	0.27 - 1.98	0.21 - 1.33

Note: Toxic indexes were calculated directly from test results.
The limits for similarity parameter were first calculated based on the range of TU sum. Then, 95% confidence interval for similarity parameter was determined.
* Reference: a: This Study; b: J. Peace, 1995; c: E. Hall, 1995; d: M. Mohsin, 1993; e: J. Prakash, 1994.

5.2.2 Prediction of joint effects

Based on the above findings, concentrations of components in mixtures that would jointly cause 50% inhibition were estimated using the appropriate QSAR models with Eq. 11 for uniform mixtures and with Eq. 12 for non-uniform mixtures. These QSAR-calculated concentrations were compared against the experimental values by utilizing all the toxicity data from binary and multi-component, uniform and non-uniform mixtures on the two cultures (refer to Table I and II). The predictions correlated well with the measured data, with $r^2 = 0.80$ for a total of 610 data points. The overall relationship between QSAR and experimental concentrations (mg/L) of the components for all the mixtures was given by:

$$\log C_{i,QSAR} = -0.159 + 1.042 \log C_{i,\,Exp.} \qquad (23)$$

$$n = 610;\ r^2 = 0.80;\ SE = 0.272;\ p = 0.0001$$

The quality of fit between the predicted and experimental concentrations for the different types of mixtures is summarized in Table IV. It can be noted that prediction for the mixtures containing chemicals not used in the derivation of the QSAR models (e.g. two types of non-uniform mixtures tested in this study totalling to 200 data points) yielded results of similar quality (Table IV). Fig. 1 illustrates this agreement between the QSAR-predictions and the measured data over nearly three orders of magnitude. The data points for each type of mixture are uniformly and randomly distributed in Fig. 1. The deviations of the points from the line of perfect prediction are due to the minor inadequacies of QSAR models, slight deviation from simple addition, as well as experimental errors. Nevertheless, this degree of prediction can be considered adequate for microbial toxicity work.

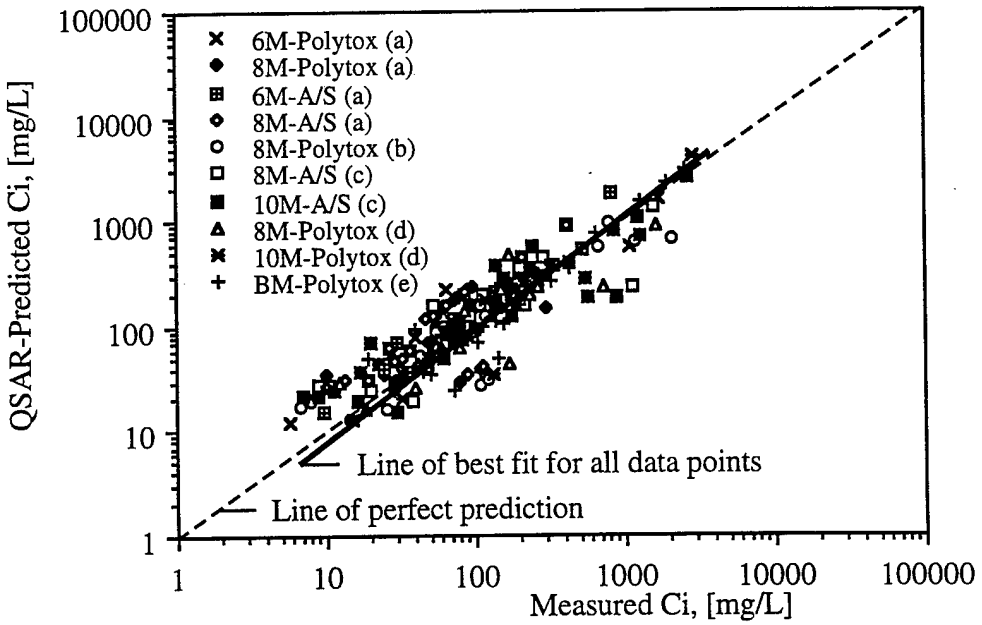

Figure 1. QSAR-Predicted vs. Measured Concentrations

Data source: (a) This study; (b) Peace, 1995; (c) Hall, 1995; (d) Mohsin, 1993; (e) Prakash, 1994

Table IV: Summary of correlation between predicted vs. measured concentrations for each type of mixtures

Mixture Type	Test Cultures	Ref.*	Parameters used in analysis				
			Slope	Intercept	r^2	Std. error	N° of data pts.
Non-uniform:							
6-chemical, non-uniform	Polytox	a	1.16	-3.52	0.89	162.4	36
8-chemical, non-uniform	Polytox	a	1.12	-6.13	0.85	86.8	64
6-chemical, non-uniform	A/S	a	0.48	39.29	0.87	82.2	36
8-chemical, non-uniform	A/S	a	0.45	28.45	0.82	42.9	64
8-chemical, non-uniform	Polytox	b	1.29	-3.75	0.65	267.9	48
Uniform:							
8-chemical, uniform	A/S	c	1.10	15.16	0.66	272.9	48
10-chemical, uniform	A/S	c	0.99	20.40	0.83	222.9	100
8-chemical, uniform	Polytox	d	1.57	-78.04	0.76	211.0	48
10-chemical, uniform	Polytox	d	0.74	85.77	0.85	212.6	100
Binary:							
Binary, non-uniform	Polytox	e	0.84	18.70	0.99	34.0	66

* References: a: This Study; b: J. Peace, 1995; c: E. Hall, 1995; d: M. Mohsin, 1993; e: J. Prakash, 1994.

Factor of error, FE, was used to evaluate the quality of this prediction. Here, FE is defined as the ratio of the predicted concentration to the measured concentration; if the ratio is less than one, then its inverse is used. The individual FE values ranged from 1.00 to 4.79 for the 610 data points, with 68% less than 2.0. The average factor of error for each type of mixtures varied from 1.34 to 2.16, and the overall average factor of error of the prediction was 1.82. Considering the similarity of the quality of fit of Eq 23 with that of the single chemical QSAR models, Eq 13-22, it can be inferred that the predictive ability of the proposed approach is comparable to that of the QSAR models. This further supports the premise that the chemicals assayed here do act by simple addition.

5.3 Comparison of Toxicity Prediction Models Reported in the Literature

Two Structure Activity Relationship (SAR) and two Property Activity Relationship (PAR) models for predicting toxicity of synthetic organic chemicals (SOCs) to activated sludge microorganisms are summarized and compared. The SAR models were developed using solvatochromic parameters and molecular connectivity indices; the PAR models, using octanol-water partition coefficient and aqueous solubility. Experimental data on sixteen chemicals determined in this research and not utilized in developing the above models are used to compare and evaluate the predictive ability of these SAR/PAR models.

5.3.1 SAR/PAR Models For Microbial Toxicity:

The basic requirement for developing SAR or PAR models is a consistent and robust training data set (Cronin and Dearden, 1995). Based on the number of chemicals tested, the test procedures used, and the stated research objectives, the studies reported by Blum and Speece (1990), Tang et al., (1992) and those developed in this project can be considered internally consistent and well designed microbial toxicity data bases. They have been utilized by their respective authors to derive SAR and PAR models using solvatochromic parameters, octanol-water partition coefficient, and aqueous solubility. These are briefly described below:

5.3.1.1 Solvatochromic parameters, V_I, π^*, α_m and β_m

The use of solvatochromic parameters in structure activity studies was pioneered by Kamlet and coworkers. Their SAR models were based on Linear Solvation Energy Relationships (LSER) where four solvatochromic parameters are used in tandem: intrinsic molar volume, V_I, polarity/polarizability, π^*, and hydrogen bond donor acidity, α_m, and basicity, β_m. The first parameter, V_I could be calculated from the molecular structures; the other three parameters have to be determined experimentally or estimated using ground rules and from related chemicals. Solvatochromic values for about 300 chemicals could be found tabulated in several of the works of Kamlet and coworkers (e.g. Kamlet et al., 1983). Hickey and Passino-Reader (1991) have recently presented a compilation of ground rules for the estimation of these parameters.

Blum and Speece (1990) have reported toxicity data and SAR models for activated sludge (as well

as methanogens, nitrifiers, and Microtox, a commercial microbial test culture). The following LSER-based SAR model for activated sludge was reported in that study (where the IC50 values were in μM/L; these have been converted to mM/L in this study):

$$\log IC50\ [mM/L] = 2.24 - 4.15\ V_I/100 + 3.71\beta_m - 0.41\ \alpha_m \qquad (24)$$
$$n = 52;\ r^2 = 0.92$$

The training data set used by Blum and Speece (1990) included eight of the 16 testing set of chemicals assayed in this study. Therefore, those eight chemicals were deleted from their data set to derive an LSER-based model in this study using the remaining 44 chemicals as the training set. The resulting model is similar in form and quality to their original one:

$$\log IC50\ [mM/L] = 1.99 - 3.74\ V_I/100 + 3.65\ \beta_m - 0.30\ \alpha_m \qquad (25)$$
$$n = 44;\ r^2 = 0.88$$

5.3.1.2 Octanol water partition coefficient, P

The use of octanol-water partition coefficient as log P in structure activity studies was pioneered by Hansch and coworkers, and has been the parameter of choice in numerous PAR studies. Listings of log P values can be found in the numerous papers published in this area. It can also be estimated using a group contribution method starting from a parent molecule; or by a fragment contribution method starting from molecular fragments (Leo et al., 1975). However, in some cases, these estimation methods may yield different results for the same chemical, depending on the starting point of the estimations. In addition, for sparingly soluble chemicals the calculated log P values may deviate significantly from the experimentally measured values.

Blum and Speece (1990) have evaluated octanol-water partition coefficient as a PAR parameter to model toxicity to activated sludge (as well as methanogens, nitrifiers, and Microtox, a commercial microbial test culture). The following log P-based PAR model for activated sludge was selected for comparison from their results (where the IC50 values were in μM/L; these have been converted to mM/L in this study:

$$\log IC50\ [mM/L] = 2.12 - 0.76 \log P \qquad (26)$$
$$n = 53;\ r^2 = 0.82$$

The training set of the above model contained five of the chemicals assayed in this study; therefore, their model was redeveloped excluding the five chemicals, to yield a very similar model :

$$\log IC50\ [mM/L] = 2.11 - 0.74 \log P \qquad (27)$$
$$n = 48;\ r^2 = 0.87$$

5.3.1.3 Aqueous solubility, S

The use of aqueous solubility in PAR applications is relatively uncommon. Since log S has been correlated with the solvatochromic parameters (e.g. Kamlet et al., 1987), with MCIs (e.g. Nirmalakhandan and Speece, 1988, 1989), and with P (e.g. Hansch et al 1968), it has been hypothesized that toxicity could be directly correlated with S (Trevizo and Nirmalakhandan, 1997). Experimentally measured S data for a large number of chemicals have been tabulated in the literature; alternatively they can be estimated for a wide range of chemicals with high degree of reliability (Kamlet et al., 1987; Nirmalakhandan and Speece, 1988, 1989).

Most of the SAR/PAR models reported in the literature, including those for toxicity, have been of a data fitting nature. End users of SAR/PAR models would be reluctant to adapt results of such studies unless the predictive ability of the models are demonstrated on external testing data sets. The primary objective of this study is to evaluate the predictive ability of the two SAR and two PAR models reported in the literature for microbial toxicity. A brief description of each of the SAR/PAR models compiled from the literature is presented below.

Trevizo and Nirmalakhandan (1997) have evaluated aqueous solubility to derive a PAR model for toxicity to activated sludge (as well as methanogens, nitrifiers, nitrobacter, Polytox, and Microtox). The following solubility-based PAR model for activated sludge was selected from their study for comparison:

$$\log IC50\ [mM/L] = -0.10 + 0.61 \log S\ [mM/L] \qquad (28)$$

$$n = 33;\ r^2 = 0.76$$

5.3.2 Summary of Models

The database consisting of the testing set of 16 chemicals and the corresponding SAR/PAR model parameters for the four models selected for comparison are tabulated in Table V. The IC50 values measured in this study, the IC50 values predicted by the respective models, and the corresponding factors of errors are presented in Table VI for the testing set of 16 chemicals. The factor of error is calculated as the ratio of the predictive IC50 value to the measured value; if the ratio is less than 1, then its inverse is used. The salient features of the four models and the quality of their predictions are summarized in Table VII. The models are compared on the basis of statistical validity, applicability and ease of use, and predictive ability.

TABLE V. SAR/PAR Parameters for Testing Set of Chemicals

ID #	Chemical	Code@	SAR/PAR Parameters*						
			Solvatochromic			Connectivity		log P	log S
			$V_i/100$	β	α	$^0\chi^v$	$^1\chi^v$		
1	2,2,2-Trichloroethanol	Alc	0.51	0.92	0.51	5.05	2.37	1.54	na
2	2,2-Dichloroethanol	Alc	0.42	0.77	0.45	3.99	2.03	0.49	na
3	1,2-Dichloro 2-methyl propane	Hal	0.64	0.10	0.00	5.47	2.72	3.03	na
4	1,2,3-Trichloropropane	Hal	0.63	0.10	0.00	5.39	3.07	1.98	1.11
5	Cyclopentane	Alk	0.50	0.00	0.00	3.54	2.50	2.05	0.34
6	1,1,2-Trichloroethane	Hal	0.52	0.10	0.00	4.68	2.51	2.05	1.52
7	1,3-Dichloropropene	Hal	0.54	0.05	0.00	4.12	2.19	1.60	na
8	m-Cresol	Aro	0.63	0.34	0.58	4.75	2.54	1.97	2.34
9	p-Cresol	Aro	0.63	0.34	0.58	4.75	2.54	1.97	2.35
10	2-Nitrophenol	Aro	0.68	0.57	0.76	4.09	2.17	1.85	1.29
11	4-Nitrophenol	Aro	0.68	0.32	0.93	4.09	2.25	1.85	2.17
12	2,4-Dinitrophenol	Aro	0.82	0.77	0.92	4.50	2.37	1.91	1.65
13	2,4-Dichlorophenol	Aro	0.72	0.18	0.78	5.94	3.09	3.07	1.44
14	2,3,4-Trichlorophenol	Aro	0.81	0.08	0.87	7.00	3.58	3.85	na
15	2,3,5-Trichlorophenol	Aro	0.81	0.08	0.87	7.00	3.57	3.85	na
16	2,4-Dinitrotoluene	Aro	0.87	0.54	0.32	5.05	2.65	2.15	na

@ Alc- alcohols; Hal- halogenated aliphatics; Alk- alkanes; Aro- aromatics

* P- octanol-water partition coefficient [-]; S - aqueous solubility [moles/L]

na Data not available

TABLE VI. Comparison of IC50 values predicted by four QSAR/QPAR models.

ID #	Chemical	Code[@]	Measured IC50 [mM/L] this study	Predicted IC50 [mM/L] and Factor of error [-]							
				Model 1		Model 2		Model 3		Model 4	
				IC50	FE	IC50	FE	IC50	FE	IC50	FE
1	2,2,2-Trichloroethanol	Alc	18.1	1922.5	106.0	44.9	2.5	8.9	2.0	-	-
2	2,2-Dichloroethanol	Alc	76.8	1233.0	16.0	80.8	1.1	55.9	1.4	-	-
3	1,2-Dichloro 2-methyl propane	Hal	5.0	0.9	5.4	2.0	2.5	0.7	7.6	-	-
4	1,2,3-Trichloropropane	Hal	3.8	1.0	3.9	2.2	1.8	4.1	1.1	3.8	1.0
5	Cyclopentane	Alk	2.3	1.3	1.8	2.8	1.2	3.7	1.6	1.3	1.8
6	1,1,2-Trichloroethane	Hal	7.7	2.6	3.0	4.7	1.6	3.6	2.1	6.7	1.1
7	1,3-Dichloropropene	Hal	3.3	1.4	2.4	8.8	2.6	8.0	2.4	-	-
8	m-Cresol	Aro	5.4	4.9	1.1	1.9	2.8	4.2	1.3	21.3	4.0
9	p-Cresol	Aro	4.8	4.9	1.0	1.9	2.5	4.2	1.1	21.6	4.5
10	2-Nitrophenol	Aro	2.3	**20.6**	9.0	**5.8**	2.5	**5.2**	2.3	**4.9**	2.1
11	4-Nitrophenol	Aro	0.9	**2.2**	2.5	**4.6**	5.1	**5.2**	5.8	**16.7**	18.6
12	2,4-Dinitrophenol	Aro	0.9	**29.7**	32.3	**3.2**	3.5	**4.7**	5.1	**8.1**	8.8
13	2,4-Dichlorophenol	Aro	0.5	0.5	1.2	0.4	1.2	0.6	1.4	6.0	13.3
14	2,3,4-Trichlorophenol	Aro	0.2	0.1	1.6	0.1	1.8	0.2	1.0	-	-
15	2,3,5-Trichlorophenol	Aro	0.1	0.1	1.4	0.1	1.5	0.2	1.1	-	-
16	2,4-Dinitrotoluene	Aro	1.1	**4.1**	3.8	**1.4**	1.3	**3.1**	2.8	-	-
	Average factor of error				12.0		2.2		2.5		6.1

[@] Alc- alcohols; Hal- halogenated aliphatics; Alk- alkanes; Aro- aromatics

* P- octanol-water partition coefficient [-]; S - aqueous solubility [moles/L]

Bold values indicate measured toxicity significantly greater than predicted toxicity

TABLE VII. Summary of Comparisons Between SAR/PAR Models

	LSER	MCI				log P	log S
	Eq (2)	Eq (3)	Eq (4)	Eq (5)	Eq (6)	Eq (8)	Eq (9)
Comparison of model features:							
Chemicals covered#	Mixed	Alc.	Aro.	Hal.	Alk.	Mixed	Mixed
N° of variables, v	3	1	1	1	1	1	1
N° of cases, n	44	14	12	14	11	48	33
N° of cases per variable, j	15	14	12	14	11	48	33
Correlation coefficient, r^2	0.88	0.83	0.66	0.79	0.75	0.75	0.76
Adjusted r^2	0.88	0.82	0.63	0.77	0.72	0.75	0.75
RMS residual	0.36	0.30	0.39	0.22	0.23	0.48	0.41
Probability, p	0.0001	<0.0001	0.0013	<0.0001	0.0006	0.0001	0.0001
Comparison of model predictions:							
N° of cases tested	16	overall for MCI models = 16				16	9
r^2 for predicted vs. measured	0.44	overall for MCI models = 0.90				0.95	ns
Probability of correlation, p	0.0053	overall for MCI models = 0.0001				0.0001	ns
Average factor of error, AFE	12	overall for MCI models = 2.2				2.5	6.1
Cases with AFE < 2.5	50%	overall for MCI models = 81%				69%	44%

Alc.-alcohols; Aro.- aromatics; Hal.- halogenated aliphatics; Alk.- alkanes
ns- statistically not significant

5.3.3 Statistical validity of the Models

In addition to the basic statistics reported for each model under the Methods and Materials Section, a key factor to be considered is the number of cases per independent variable. In linear regression analysis, the number of cases per independent variable, j, should be high enough to avoid chance correlations (Topliss and Costello, 1972). It is generally accepted that j should be at least 10, and preferably greater than 20. The two PAR models satisfy this criterion more than adequately. Of the two SAR models, the molecular connectivity-based model suffers most from this criterion because different congeneric groups of chemicals require different models to explain the variance among the cases. Due to the small number of chemicals covered by each of the equations, their j values range from 11 to 14 (Table VII). Even though the validity of these models has been documented and justified using a variety of statistical tests (Hall et al. 1996), end users may be reluctant to apply such models unless their predictive ability on a wider range of chemicals is well demonstrated. Although the LSER model given by Eq 25 covers 44 cases, it needs three independent variables to explain 88% of the variance among the cases and is thus only slightly better than the MCI models with j = 15.

The balance between number of cases covered, n, and the number of independent variables, v, required to explain a reasonable percentage of the variance among the cases is often a subject of debate. Because of the limitations of the resources available for toxicity testing on one hand and the information content of the molecular descriptors on the other, a compromise has to be made. The adjusted r^2 is a statistical measure that can compare different models with v and n combinations. Based on the adjusted r^2 values calculated in this study for the different models, all of them except the MCI model for aromatic compounds can be seen to be acceptable (Table VII)

Another consideration in regression analysis is that intercorrelation between the independent variables should be minimal when the model requires multiple variables. For the LSER model presented here with three independent variables, intercorrelation between them was found to be negligible, with a maximum r^2 of -0.29 between $V_I/100$ and β_M for the chemicals used in developing the model.

As a third consideration, linear regression models have to be statistically significant to ensure that the model is not due to chance correlations. A criterion that is often used to test for significance is the p value. It is generally accepted that $p < 0.05$ implies borderline significance; $p < 0.01$ implies significance; and $p < 0.005$, high significance. All the models evaluated in this study were found to be statistically highly significant as indicated by their respective p values (Table VII). In other words, these models encode a systematic variation of toxicity with the respective structural features or properties, and the relationship is not due to pure chance alone.

5.3.4 Applicability and Ease of use of the Models

From the end users' perspective, the model parameters should be readily available for a wide range of chemicals, error-free, and reliable; and the models themselves should be applicable to wide

range of chemicals. Based on the availability of model parameters, the molecular connectivity-based approach proved to be the most convenient because, a standard algorithm was available to calculate all the parameters starting from the molecular structures. While a computer program was used in this study to determine the MCIs, the calculations can be manually performed with ease.

Solvatochromic parameters for only 11 of the chemicals could be found from literature; values for the remaining five chemicals (ID # 1, 2, 10, 12, and 16 in Table V) had to be estimated using the rules reported in the literature (Hickey and Passino-Reader, 1991). The estimated solvatochromic values could not be verified independently. The rules for estimating the solvatochromic parameters are far from rigid and consistent; their application to "new" chemicals requires considerable chemical insight, intuition, and judgment; and several optional corrections, modifications, and adjustments have to be made as deemed necessary by the user (Hickey and Passino-Reader, 1991).

The log P values used in this PAR work are calculated rather than experimental. Due to the high uncertainties in the measured log P values and the availability of a simple algorithm for their calculation, the calculated values are recommended to assure consistent and reproducible results. However, for some chemicals, the calculated values may not yield unique values.

The S values used here are all experimental values found from the literature. Values for S were not readily available for seven of the chemicals assayed. Even for the common chemicals for which experimentally measured data exist, significant discrepancies can be noted; and the experimental errors may be large, particularly for the sparingly soluble ones which are environmentally relevant. However, since toxicity is a solubility related phenomenon, correlations with aqueous solubility may be of significance in further studies in mechanistic understanding of microbial toxicity.

In terms of applicability, the LSER model has to be rated below the MCI model. For instance, even though the training set for the LSER model had eight chlorinated phenols, its predictions for the three chlorophenols (ID # 13, 14, 15 in Table V) in the training set of this study are not better than those for the MCI model, whose training set did not include any phenols at all. For the two halogenated aliphatics tested (ID # 3, 4 in Table V), the quality of the predictions by the LSER model is inferior to that of the MCI model in spite of the fact both models were trained on several such chemicals. It is interesting to note that the MCI model predicted remarkably well for the two chlorinated alcohols (ID # 1, 2 in Table V) even though its training set did not contain any. From these results it is deduced that the MCI approach can perform well even for molecules with multiple atomic constituents as long as those constituents are adequately represented individually in the training set. Similar results were found in other MCI-based QSAR studies on aqueous solubility, Henrys Constant etc Nirmalakhandan (1988).

5.3.5 Predictive ability

The predictive ability is compared on the basis of two factors: first, the degree of agreement between the predicted and measured IC50 values; and second, the factor of error in the predictions.

Considering the reproducibility of IC50 values measured by the respirometric method for microbial cultures; the ease of use of SAR/PAR models; and the quality of fit of the state-of-the-art SAR/PAR models, it is proposed that an average factor of error, AFE, of 2.5 be considered an acceptable criterion.

The overall quality of the predictions by the four models is illustrated in Fig 2. Based on the statistically significant ($p = 0.0001$) correlation between the measured and the predicted IC50 values, the log P method ranks best ($r^2 = 0.95$) followed closely by the MCI model ($r^2 = 0.90$). The LSER model was not satisfactory ($r^2 = 0.44$) with a marginally significant ($p = 0.0053$) correlation, although it covered only nine chemicals. No statistically significant correlation was found between the measured IC50 values and those predicted by the log S model. The breakdown of the latter two models may be due to the inadequacies of the models themselves or/and to the fact that the model parameters (i.e. the solvatochromic parameters and S) themselves may be erroneous. At this point it is not possible to distinguish between the two.

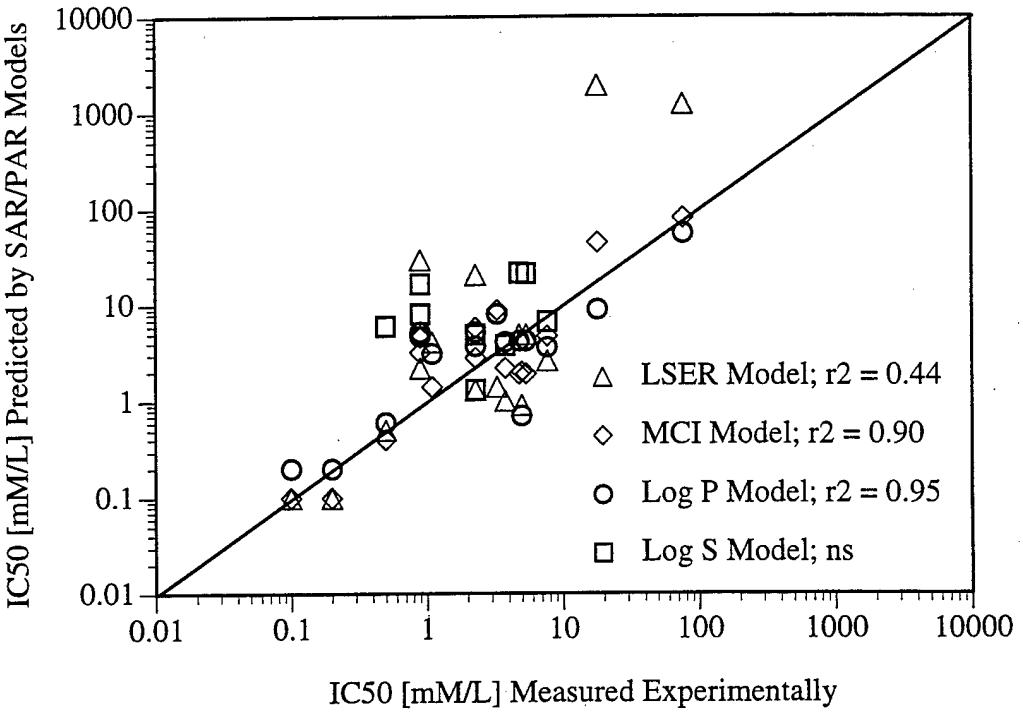

Figure 2. Comparison Between Experimental and Predicted IC50 Values
Note: r^2 for log S model was not significant.

Based on the overall AFE, the MCI models and the log P model can be considered to be good predictors of microbial toxicity; their predictions for 75% of the test chemicals are within a factor of error of 2.5. The predictions of the LSER and log S models for 50% of the test chemicals are above the acceptable factor of error (Table VII). All four models identified the nitro-aromatic chemicals, (ID # 10, 11, 12, and 16 in Table V) to be more toxic than the predictions; this is in agreement with the other researchers in that these chemicals are known to act reactively. All four models predict poorly for 2,4-dinitrophenol with AFE > 2.5, again confirming its highly reactive toxic mechanism (Fig 3).

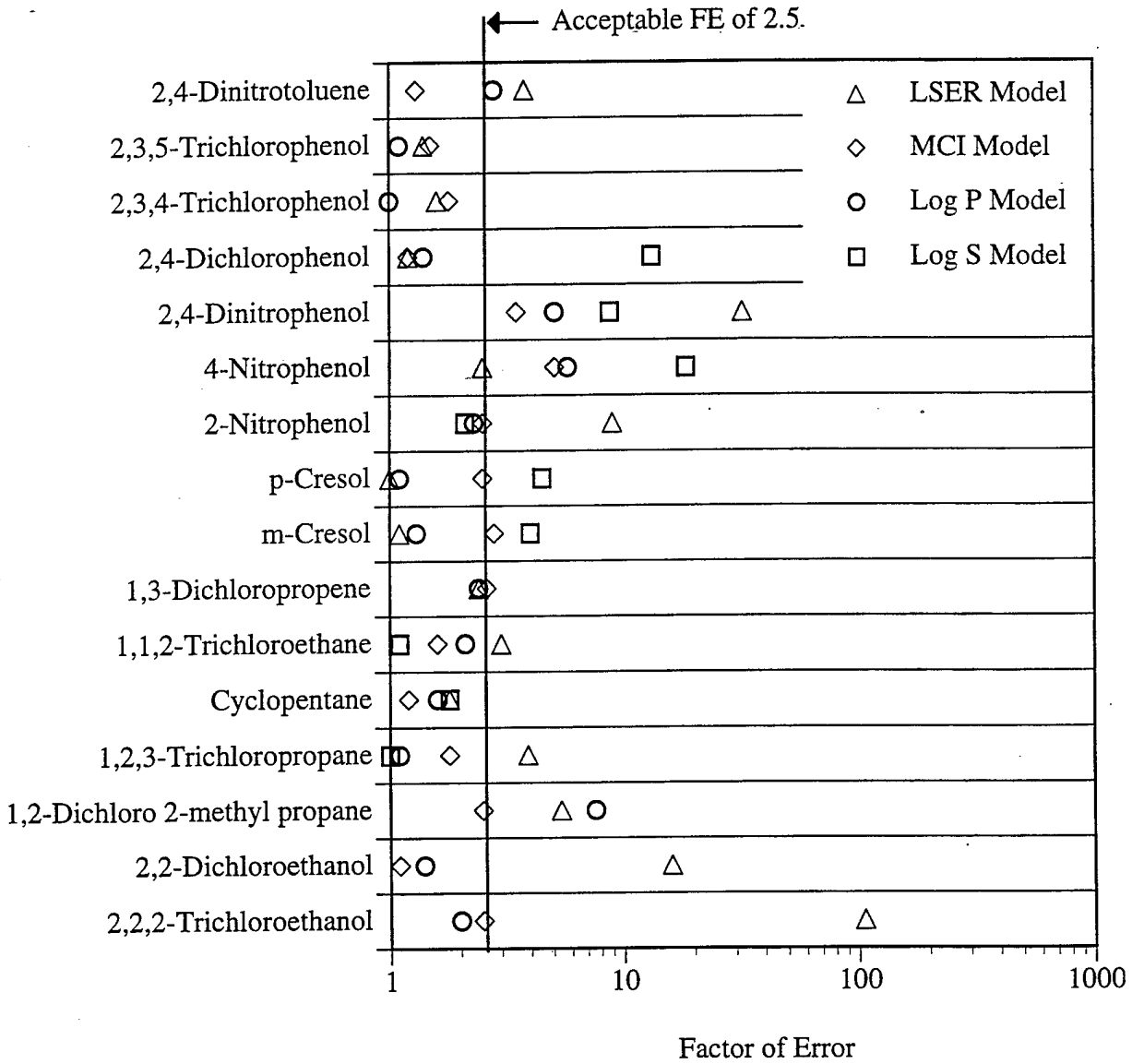

Figure 3. Comparison Between Predictive Factor of Error by Four SAR/PAR Models

The overall quality of the LSER method is particularly impaired by 2,2,2-trichloroethanol and 2,2-dichloroethanol. Since its predictions for the remaining chemicals are acceptable and comparable to the predictions made by the other models, the estimated solvatochromic values for these two chemicals are suspicious. The β values of 0.92 and 0.77, in particular, "appear" to be too high. These values were estimated (by Hickey, 1997) using the ground rules (Hickey and Passino-Reader, 1991), by adding contributions for two aliphatic carbons, an aliphatic hydroxyl group and 3 or 2 aliphatic chlorines. These values may have to be corrected by allowing for diminishing contributions by the successive additions of chlorines or by using a leveling factor of 0.8 to 0.9. Even with such adjustments, the predictions for these two chemicals are not acceptable. Anomalies such as this and similar difficulties in establishing the solvatochromic parameters have greatly discouraged the application of the LSER approach by a wider range of researchers.

6.0 CONCLUSIONS

Joint effects of binary and multi-component, uniform and non-uniform mixtures assayed in our microbial toxicity studies were found to be simply additive, or essentially simply additive. These results are in agreement with the conclusions reported in the literature on fish toxicity studies. Using QSAR models to predict single chemical toxicity and assuming perfect simple additivity, concentrations of the components in mixtures that would cause 50% inhibition were predicted. These predicted concentrations agreed well with the measured values over nearly three orders of magnitude with $r^2 = 0.80$ at $p = 0.0001$ for 610 sets of data points from 40 different mixtures on two different microorganisms. The overall average factor of error of these predictions was 1.82. The results of this study provide an impetus to utilize the large number of single chemical QSAR models reported in the literature by other researchers in predicting joint effects in the aquatic toxicology and ecotoxicological fields.

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